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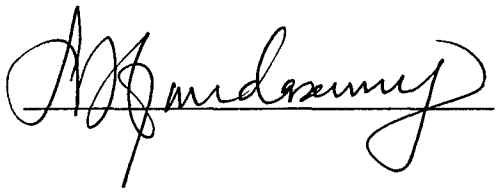

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13. ABSTRACT ***(Maximum 200 Words)***

To determine the feasibility of endothelial cell-based gene therapy for metastatic breast cancer, we investigated the optimal dose, toxicity, and efficiency of incorporation of intravenously (IV)-administered, human interleukin-2 gene-modified murine microvascular endothelial cells (hIL-2/MECs) into individual metastatic foci of breast cancer. Following IV injection of one or three doses of 10^5, 10^6, or 10^7 hIL-2/MECs to BALB/c mice bearing pulmonary metastasis of breast cancer, various tissues from the animals were examined at varying intervals for the presence and expression of hIL-2 gene by DNA polymerase chain reaction (PCR) and reverse transcriptase (RT)-PCR techniques.

In mice treated with a single IV injection of 10^5 hIL-2/MLECs, 2, 5, and 2% of lung metastases obtained were hIL-2 positive by both DNA PCR and RT-PCR on day 7, 14, and 21, respectively. Animals given a single or multiple IV injections of 10^6 or 10^7 hIL-2/MLECs died from toxicity. In contrast, three sequential IV injections of 10^5 hIL-2/MLECs (at 3-day interval) had no deleterious effects in the animals. Eighty, 90, and 30% of lung metastases recovered from these mice were hIL-2 positive on day 7, 14, and 21, respectively. No hIL-2 gene was detected in all other tissues of these mice or in control tumor-bearing mice.

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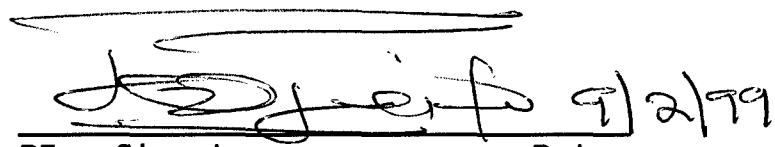

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1. INTRODUCTION

Breast cancer is the most common female malignancy in North America (1). This disease is estimated to affect 1 of 9 women (2), and is associated with substantial morbidity and mortality (1). While early detection and treatment have led to significant improvements in cancer-related mortality and quality of life for women with breast cancer, recurrence and metastatic dissemination of the tumors still account for a significant morbidity and mortality in patients. Effective means of treating this subset of patients remains elusive. A novel approach to the problem of recurrent or metastatic cancer involves the activation of potent immune responses that are capable of specifically destroying tumor cells. Transgenic immunotherapy, as the term implies, refers to the insertion of cytokine genes into cells in order to activate anti-tumor immune responses. Moreover, this approach is intended to avoid the dose-limiting toxicities that have impeded the application of otherwise very promising cytokine therapies. The goal of this research is to develop an effective and safe gene therapy for invasive breast cancer. The objectives of the research are (1) to determine whether intravenously (IV) administered endothelial cells expressing exogenous cytokine gene(s) can selectively migrate into pulmonary metastases of breast tumors, express the cytokine transgene at the metastatic sites, and elicit anti-tumor immune responses, and (2) to determine the safety of IV-administered, genetically-modified endothelial cells. This report covers the investigation of (a) the efficiency of hIL-2/MLEC incorporation at multifocal tumor sites, and (b) the optimal dose and toxicity of IV administration of hIL-2/MLECs.

2. BODY

2.1 Specific Aims and Statement of Work

The specific aims of this research are (1) To determine (a) whether IV-injected, interleukin-2 gene-modified murine lung endothelial cells (IL-2/MLECs) can target sites of pulmonary metastases of breast cancer, and (b) how well IL-2/MLECs can express the IL-2 transgene at the metastatic sites; (2) To determine whether the expression of hIL-2 transgene at the local site of pulmonary metastases will induce an anti-tumor immune response. The approved Statement of Work is as follows:-

Task 1: **Months 1-24.**

Determine (a) whether IV-injected, interleukin-2 gene-modified murine lung endothelial cells (IL-2/MLECs) can target sites of pulmonary metastases of breast cancer, and (b) how well IL-2/MLECs can express the IL-2 transgene at the metastatic sites.

a. Mouse lung endothelial cells (MLECs) will be isolated and enriched using FDG-FACS. The cells will be transduced with a retroviral vector containing human IL-2 gene.

b. Efficiency of IL-2/MLEC incorporation at different tumor sites:
   - Co-localization of IL-2/MLEC and tumor in animals: three experiments; 40 animals per experiment.

c. Determination of toxicity of IV IL-2/MLEC administration:
- Acute toxicity following a single dose of 10^5 IL-2/MLEC administration
- Cumulative toxicity following 3 IV injections of 10^5 IL-2/MLECs spaced 3-4 days apart.
Three experiments; 40 animals per experiment.

d. Optimization of IL-2/MLEC incorporation in tumor sites:
-Tumor-bearing animals will receive three IV injections of IL-2/MLECs closely (3-4 days) or widely (5-7) apart. Expression of IL-2 transgene at the metastatic sites determined by RNA PCR amplification of human IL-2 in discrete individual metastases. Four experiments; 40-50 animals per experiment will be performed.
-Comparison of the relationship between different administration schedules with the number of cells incorporated at sites of tumor metastases will be determined. Two experiments; 40 animals per experiment will be performed.

Task 2: **Months 24-36.**

Determine whether the expression of hIL-2 transgene at the local site of pulmonary metastases site will induce an anti-tumor immune response.

Groups of experimental of experimental and control animals will be sacrificed weekly to monitor hIL-2 expression in the lungs, quantitate metastases, and to assess lung tumor response to IL-2/MLEC treatment. One group of the experimental and control animals will be observed over time for survival. Survivors will receive additional MFP injection of 4T1 cells to determine their ability to reject tumor re-challenge.

2.2 Major Research Accomplishments

Overview

We have made significant progress in studies outlined in task 1 during the past year. Specifically, we successfully generated interleukin-2 gene-modified murine lung endothelial cells (IL-2/MLECs) which were used to determine the efficiency and optimal dose of hIL-2/MLEC incorporation into sites of tumor metastases. We have also evaluated the toxicity of systemic administration of hIL-2/MLECs.

Methods

Endothelial cells harvested from the lungs of BALB/c mice were transduced with a retroviral vector containing the rhIL-2 gene under the transcriptional control of a CMV promoter. These cells (hIL-2/MLECs) secrete 76 ng (1000 IU)/10^6/24h) of recombinant human interleukin-2 *in vitro*.

Tumor-bearing mice were developed by injecting of 10^5 4T1 tumor cell line (derived from a mammary tumor in BALB/c mouse) into the mammary fat pad of syngeneic mice. Two weeks later, at the time when the earliest pulmonary metastases are observed, the primary tumors were excised, and one or three doses of hIL-2/MECs were intravenously injected via the tail vein. Multiple injections of hIL-2/MECs were spaced 3 days apart. Beginning on day 7 after the last administration of hIL-2/MECs, at the time when significant accumulation of genetically-modified endothelial cells

(GMECs) and expression of exogenous gene occurs at sites of angiogenesis (3), groups of animals were sacrificed at varying intervals (7, 14, and 21 days), to follow the fate of injected cells over time. Heart, lungs, liver, spleen, long bones, and brain tissues were removed from the animals and examined for the presence and expression of hIL-2 gene using DNA polymerase chain reaction (PCR) and reverse transcriptase (RT-PCR) techniques.

Results and Discussion

In mice treated with a single IV injection of 10^5 hIL-2/MLECs, 2, 5, and 2% of their lung metastases examined were hIL-2 positive by both DNA PCR and RT-PCR on day 7, 14, and 21, respectively (Table 1).

Table 1: Percentage of pulmonary metastases targeted by hIL-2/MLECs at varying intervals after a single intravenous injection of the GMECs

Study group	Treatment	# metastases per mouse	% of metastases positive for hIL-2 gene		
			Day 7	Day 14	Day 21
1	Tumor + hIL-2/MLECs	>200	2	5	2
2	Tumor + Neo/MLECs	>200	0	0	0
3	Tumor alone	>200	0	0	0
4	hIL-2/MLECs	0	0	0	0
5	None	0	0	0	0

Attempts to improve the targeting efficiency by increasing the dose of the implanted hIL-2/MLECs from 10^5 to 10^6 and 10^7 were unsuccessful. On the contrary, many of the animals that received single or multiple IV injections of 10^6 or 10^7 of hIL-2/MLECs became ill and subsequently died, indicating that this large inoculum of GMECs was toxic to the animals. One explanation for this poor targeting efficiency is the heterogenous angiogenic behavior of the individual tumor metastases. That is, in certain instances, the IV-injected hIL-2/MLECs seeded growing tumors at an optimal moment for incorporation and growth within the developing tumor vasculature. Other metastases, perhaps too small to sustain very active angiogenesis, could not promote sufficient GMEC incorporation. In addition, administration of a single bolus injection of GMEC is unlikely to target every tumor deposit present at the moment of injection. Another factor affecting the efficiency of GMEC-targeting of tumor metastases is the ongoing seeding of the lungs with tumor

cells still in circulation or tumor cells dislodged from other sites at the time of hIL-2/MLECs treatment. This results in repetitive seeding of the lungs, making assessment of the efficiency of GME- targeting difficult. For these reasons, we hypothesized that the kinetics of GMEC administration will determine, to a large extent, both targeting efficiency and extent of GMEC incorporation at a particular tumor deposit. We further hypothesized that small multiple injections of small inoculum of GMECs would reduce their toxicity in the animals. To test this hypothesis, we administered 3 injections of 10^5 hIL-2/MLECs spaced 3 days apart. As predicted, multiple injections of small inoculum of hIL-2/MLECs increased the safety and the targeting efficiency at multifocal tumor sites. Three sequential IV injections of 10^5 hIL-2/MLECs (at 3-day interval) had no deleterious effects on the animals. Eighty, 90, and 30% of the individual lung metastases recovered from this group of tumor-bearing mice were positive for hIL-2 positive on day 7, 14, and 21 (Table 2).

Table 2: Percentage of pulmonary metastases targeted by hIL-2/MLECs at varying intervals after three intravenous injections of the GMECs

Study group	Treatment/	# metastases per mouse	% of metastases positive for hIL-2 gene		
			Day 7	Day 14	Day 21
1	Tumor + hIL-2/MLECs	>200	80	90	30
2	Tumor + Neo/MLECs	>200	0	0	0
3	Tumor alone	>200	0	0	0
4	hIL-2/MLECs	0	0	0	0
5	None	0	0	0	0

We did not detect hIL-2 gene in any other tissue obtained from all hIL-2/MLECs-treated mice. As shown in Tables 1 and 2, all tissues obtained from vector (Neo/MLEC, no hIL-2 gene insert) -treated mice were hIL-2 gene negative. Furthermore, we did not observe any tumor in normal mice which received the same three IV injections of hIL-2/MLECs, nor we did not find any significant statistical difference in the number of metastatic foci seen in the lungs of tumor-bearing mice with and without hIL-2/MLECs treatment, suggesting that systemic administration of gene-modified endothelial cells did not promote the formation of tumors in these animals.

2.3 Plans for the Future

In the coming year, we plan to complete all studies under task 1. Specifically, further experiments will be performed to:- 1) compare closely (3-4 days) with widely (5-7) spaced IV injections schedule of hIL-2/MLECs; 2) determine whether IV-injected hIL-2/MLECs can lodge within an inactive or a physiologically active site in mice, and (3) determine whether IV-administration of hIL-2/MLECs can promote tumor formation in mice. Also, we plan to investigate a) whether the expression of hIL-2/MECs at metastatic sites can induced an anti-tumor response that will abrogate tumor metastasis, b) the nature, level, and duration of anti-tumor immune response that is induced at the local tumor site; and c) the ability of microvascular endothelial cells (MECs) expressing interleukin-12 (IL-12) transgene or herpes simplex thymidine kinase (HSV-TK) gene to inhibit the growth of established breast cancer metastases mice.

3. Key Research Accomplishments

We have:

1) Isolated pure population of lung endothelial cells from BALB/c mice. The cells have been transduced with a retroviral vector containing human IL-2 gene and high expressing clones have been isolated and fully characterized.
b) Determined the efficiency of hIL-2/MLEC incorporation into sites of breast cancer metastasis.
c) Optimized hIL-2/MLEC incorporation into sites of pulmonary metastasis of breast cancer.
d) Determined acute and cumulative toxicity of IV-administered hIL-2/MLECs.

4. Reportable Outcomes

None

5. Conclusions

These results demonstrate that (1) three IV injections of 10^5 hIL-2/MLECs, given at 3- day intervals, efficiently target tumor metastases, (2) genetically-modified microvascular endothelial cells can express interleukin-2 transgene at the local site of breast cancer metastasis, and (3) multiple IV injections of 10^5 hIL-2/MLECs (given at 3- day intervals) can be safely administered to mice. These results will enable us to proceed to determine whether IV injections of hIL-2/MLECs can abrogate breast cancer metastases and prolong the survival of the tumor-bearing mice.

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7. Appendices None

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